Cellulase Production and Ammonia Metabolism in *Trichoderma reesei* on High Levels of Cellulose

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Summary

Trichoderma can be cultured in stirred-tank fermentors on high (8%) cellulose concentrations without increasing the salt concentration of the medium when NH_4OH is used to control pH and as a nitrogen source. Approximately 90% of the ammonia consumed by the organism can be added as NH_4OH. The advantage of using high concentrations of cellulose is that culture filtrates with greater cellulase activity are obtained. The advantage of a low salts medium is that unwanted solutes in the final enzyme preparation are reduced. The appearance of cellulase in the medium occurs later than net ammonia uptake so that only 20% of the final amount of cellulase has appeared when 80% of the maximum amount of ammonia has been consumed.

INTRODUCTION

Mandels and Reese[1] developed a medium that has been used successfully for the production of cellulase and other enzymes of microbial origin. This medium was devised for flask culture and contained $(NH_4)_2SO_4$ and urea as the nitrogen sources and a high level (15m*M*) of KH_2PO_4 as a buffer. During growth ammonia is consumed and acid conditions develop in the medium. With cellulose concentrations in excess of 1% the pH of the medium becomes so low as to inactivate the cellulase enzymes[2] and to bring the growth nearly to a halt.[3] Higher cellulose concentrations (1.5%) have been used successfully in flask culture by increasing the level of phosphate buffer.[4] However, in fermentors equipped with pH control devices, the use of salts as buffers is not necessary. Much greater cellulase production is possible in fermentors than in flask cultures by using higher cellulose concentrations while controlling the pH above 3.0.[5] At higher cellulose concentrations it is necessary

Biotechnology and Bioengineering, Vol. XXI, Pp. 181–191 (1979)
 0006-3592/79/0021-0181$01.00

to increase the amount of nitrogen. This has been done by increasing the level of peptone and/or $(NH_4)_2SO_4$,[5,6] or by increasing all of the salts for growth on high concentrations of cellulose[7] and glucose.[8] One way of adding nitrogen without increasing the salt concentration is to control pH by the addition of NH_4OH. This paper explores the feasibility of culturing the cellulolytic fungus *Trichoderma* on cellulose at concentrations up to 8% in a medium having a salt concentration lower than the original Mandels-Reese medium.

MATERIALS AND METHODS

Cultural

The organism used in this study was *Trichoderma reesei* (formerly assigned to *T. viride*) QM 9414.[9] Stock cultures were stored at 24°C on potato dextrose agar slants. The fungus was cultured in a 14 liter stirred-tank fermentor (New Brunswick Scientific, Edison, N.J.) with a 10 liter working volume (44 cm high by 22 cm in diam). The vessel was sparged with air at a rate of 2.0 to 2.5 liter/min (3.5 liter/min was used in a glucose fermentation). The apparent dissolved oxygen was maintained above 40% saturation (of air) by varying the agitation (three impellers 8 cm in diam) from 400 to 800 rpm during the course of the fermentation. pH was controlled not to fall below 3.5 by the addition of 2*N* NH_4OH. Foam was controlled by the addition of silicone antifoam SAG 100 (Union Carbide Corp., Tarrytown, N.Y.). Temperature was maintained at 28°C. The growth media contained varying levels of a commercially ball-milled cellulose (BW 200) prepared from purified spruce pulp (Brown Co., Berlin, N.H.). The pH of the fermentor media after autoclaving was 4.5 and was adjusted to pH 5.5 by the addition of approximately 2.5 mmol NH_4OH/liter. A two-stage inoculum patterned after the method of Nyström and Allen[10] was used: The first stage was cultured in shake flasks containing 100 ml Mandels-Reese medium (hereafter referred to as standard medium) with 1% BW 200, 0.1% proteose peptone, and 0.1% Tween 80 for three days at 28°C; the first stage was used at 10% to inoculate Fernbach flasks containing 1 liter of the same medium except that 2% BW 200 was used. The second-stage inoculum was cultured at 28°C on a rotary shaker for 24 hr and then transferred to the fermentor at either 10 or 20%.

Assays

Cellulase activity was measured by the release of reducing sugar from Whatman No. 1 filter paper strips.[11] β-Glucosidase activity

was measured by the release of glucose from a cellobiose solution.[5] One unit (U) is the amount required to release 1 μmol glucose/min. Enzyme assays were performed on culture filtrates at pH 4.8 in 0.05m*M* sodium citrate buffer at 50°C (mycelial associated enzyme was not assayed). Protein in the culture filtrates was assayed by the Folin method[12] after precipitation in 5% trichloroacetic acid. The exit gas was analyzed for CO_2 with an infrared analyzer and O_2 by a paramagnetic analyzer (Mine Safety Products Co., Pittsburgh, Penn.) to calculate respiration rates. The amount of NH_4^+ present in the culture filtrate was determined by measuring the ammonia liberated with an ammonia electrode (Orion Research Inc., Cambridge, Mass.) after adjusting the pH of the sample to 12.

Saccharification Potential

Enzyme strength was evaluated by incubating culture filtrates at various dilutions with 15% BW 200, pH 4.8, in 125 ml flasks (50 ml reaction volumes) at 50°C with constant rotary shaking. Samples were taken periodically, heated in boiling water for 5 min to stop the reaction, centrifuged, and the supernatant fluid analyzed for total carbohydrate by the phenol–H_2SO_4 method.[13] Qualitative analyses of the sugars were performed on a high-performance liquid chromatograph (Waters Associates, Milford, Mass.) using a μ-Bondapak carbohydrate analysis column with acetonitrile: H_2O (75/25) eluting solvent.

RESULTS

NH_4OH as pH Controllant and Nitrogen Source on Elevated Levels of Cellulose

Fermentations with concentrations of 2 to 8% BW 200 were carried out (Fig. 1) using the standard medium but omitting urea to simplify NH_4^+ determinations and doubling the amounts of the trace metals. Proteose peptone and Tween 80 (both at 0.1%) were included, and a 10% inoculum was used. In all fermentations the pH fell to the control point (pH 3.5) within 12 to 16 hr, after which 2*N* NH_4OH was automatically added to control pH and to serve as an additional nitrogen source. During the period of pH control, NH_4^+ decreased in the medium to a level proportional to the amount of cellulose initially present (Fig. 1(b)). In the 8% cellulose culture the NH_4^+ might have been depleted. Even though the level of NH_4^+ residing in the medium fell, most of the nitrogen was supplied as NH_4OH; for example, in the 6% cellulose fermentation a total of

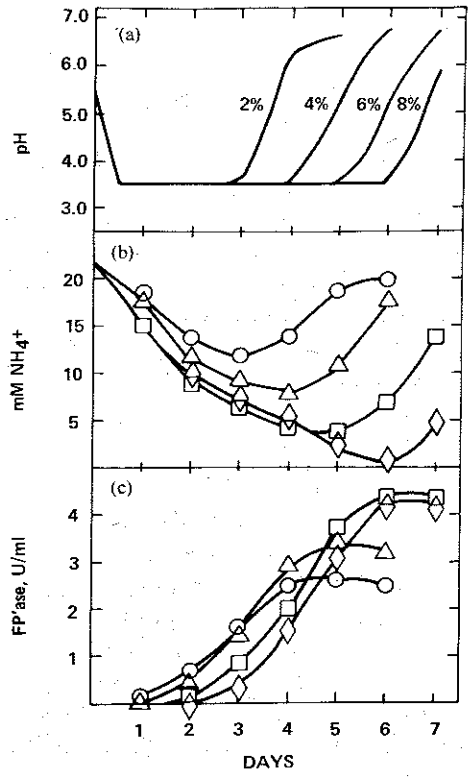

Fig. 1. Fermentations on (○) 2%; (△) 4%; (□) 6%, and (◇) 8% cellulose using the standard salts medium. (a) pH profiles; (b) m*M* NH_4^+ concentration; (c) cellulase activity (filter paper cellulase).

120 mmol NH_4^+/liter was added as NH_4OH. At the end of the pH control period the pH was allowed to rise naturally (Fig. 1(a)) and in each case the rise in pH corresponded with an increase in NH_4^+ in the medium. This occurred in a glucose culture as well (Fig. 2). Thus it appeared that at the end of the acid phase of the fermentation, the organism released NH_4^+ into the medium.

The amount of cellulase produced increased linearly with increasing cellulose concentration up to 6% (Fig. 1(c)). The amount of cellulase produced on 8% cellulose was about the same as on 6%. On 8% cellulose growth was slower than the other fermentations with the peak respiratory activity (12 mmol CO_2/liter hr) being less than that on 6% cellulose (13 mmol/liter hr). The same observations of slower growth and low cellulase yields on 8% cellulose were made when the organism was cultured on a medium having 34m*M*

NH_4^+ initially and the minimum NH_4^+ concentration was 17m*M*. Therefore the lower yield of cellulase on 8% cellulose was not due to nitrogen limitation. Maximum β-glucosidase levels (not shown) varied from 1.0 U/ml on 2% cellulose to 1.6 U/ml on 6% cellulose.

The carbon-to-nitrogen ratios were calculated from the amount of NH_4OH added to control pH, the maximum NH_4^+ removed from the medium, the amount of carbon and nitrogen supplied by peptone and carried over in the inoculum, and the amount of carbon as cellulose. Assuming that all the peptone was consumed, the C:N ratios should reflect the actual amount of nitrogen consumed by the organism and not simply the amount originally supplied in the medium. The C:N ratios were around 12 for 2% to 6% cellulose and for 6% glucose, but was 16.5 for the 8% fermentation.

To reduce unnecessary salts, the KH_2PO_4 of the standard medium was replaced by a low level of K_2SO_4, and the $(NH_4)_2SO_4$ by

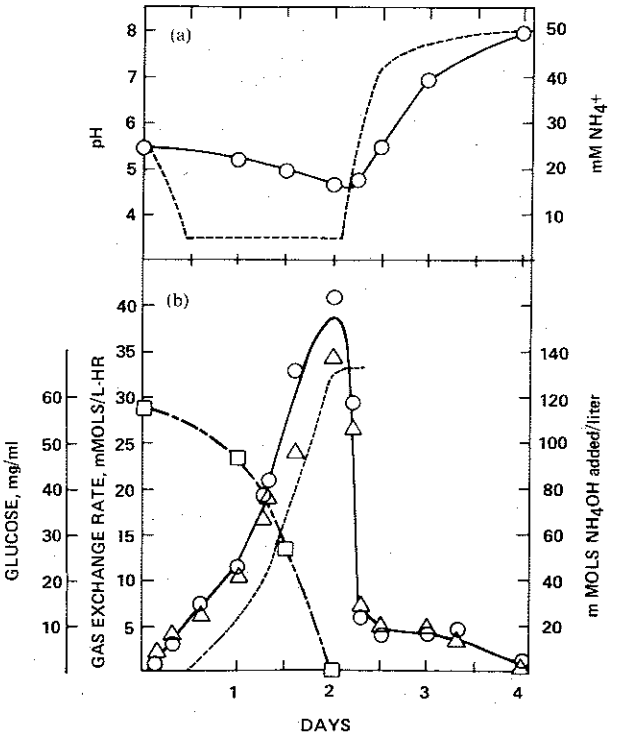

Fig. 2. Fermentation profile from 6% glucose with standard salts medium. (a) (---) pH, (○) NH_4^+ remaining in the medium; (b) (---) NH_4OH added, (□) glucose remaining in medium, respiration rates based on (○) CO_2 and (△) O_2.

$NH_4H_2PO_4$. Urea was omitted. The revised medium had NH_4^+ as the principal cation and PO_4^{3-} as the principal anion (Table I). In the original medium K^+ and SO_4^{2-} were present in relatively high concentrations. Although total salt content was reduced by only 25%, the revised medium maintained a higher NH_4^+ level during fermentation.

The revised medium was first evaluated using 6% cellulose, and the cellulase productivity was comparable to that on the standard medium (4.5 filter paper (FP) U/ml). Although approximately the same amount of NH_4OH was used, the amount of NH_4^+ in the medium remained at a higher level—9m*M* was the minimum compared to 4m*M* with the standard medium. With 8% cellulose in the revised medium and a 10% inoculum, cellulase yield and respiration rate again were low. With a 20% inoculum cellulase production increased more rapidly and reached a higher level (Fig. 3). The final

TABLE I
Comparison of Components of Mandels–Reese Medium and a Minor Revision Thereof

Component	Standard	Revised
	(m*M*)	
NH_4^+	21.0	21.0
PO_4^{3-}	15.0	21.0
K^+	15.0	3.5
SO_4^{2-}	13.0	4.2
Urea	5.0	0
Ca^{2+}	2.7	2.7
Mg^{2+}	2.5	2.5
Fe^{2+}	0.018	0.036
Mn^{2+}	0.009	0.018
Zn^{2+}	0.012	0.024
Co^{2+}	0.009	0.018
Total	74.2	55.0
Compound	**Standard**	**Revised**
	(g/liter)	
Proteose peptone	1.0	1.0
$(NH_4)_2SO_4$	1.4	–
KH_2PO_4	2.0	–
Urea	0.3	0
$CaCl_2$	0.3	0.3
$MgSO_4$	0.3	0.3
$NH_4H_2PO_4$	–	2.4
K_2SO_4	–	0.3

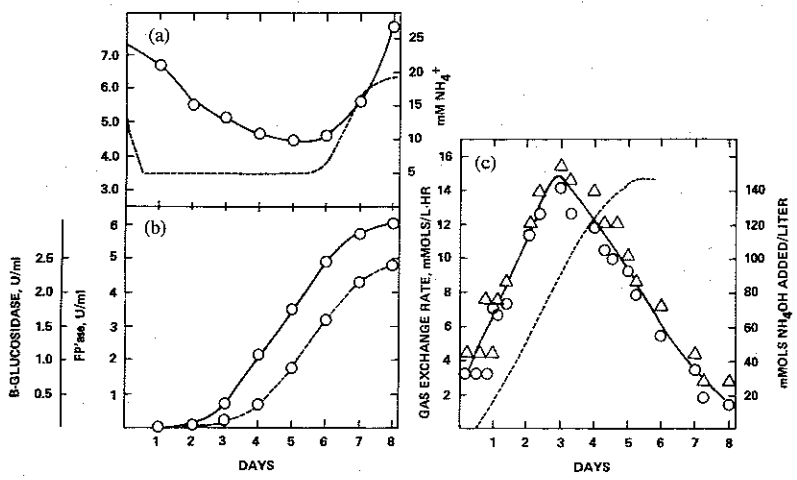

Fig. 3. Fermentation profile on 8% cellulose with revised medium. (a) (---) pH, (○) NH_4^+ remaining in medium, (b) (—) cellulase activity, (---) β-glucosidase activity; (c) (---) NH_4OH added, respiration rates based on (○) CO_2 and (△) O_2.

enzyme yields were around 6.0 U/ml for filter paper cellulase and 2.5 U/ml for β-glucosidase. The culture filtrate had a final concentration of 10 mg protein/ml. As was found previously, the decrease of NH_4^+ in the revised medium was less than on the standard medium (Fig. 2(a)). The respiration rate peaked at approximately 15 mmol CO_2–O_2/liter hr at three days and declined over the next five days (Fig. 3(c)). This slow decline was in marked contrast with respiration on glucose, which, after reaching its maximum, fell to a low, basal rate in 6 hr (Fig. 2). Presumably the gradual decline on cellulose was due to the multiplicity of the substrate—the easily degraded portions were assimilated first, leaving an increasingly resistant residue. The C:N ratio for 8% fermentations was 14.6.

Relationship Between NH_4^+ Uptake and Cellulase Appearance

Little cellulase appeared in the culture filtrate until most of the ammonia was consumed (Fig. 4). A significant amount of cellulase accumulated in the medium after uptake of NH_4^+ had ceased and the concentration of NH_4^+ was increasing in the medium. When 80% of the ammonia was taken up, only about 20% of the final level of cellulase was detected in the filtrate. This general pattern of 80%–20% crossover was found for all fermentations using BW 200 regardless of the cellulose concentration or the type of medium used.

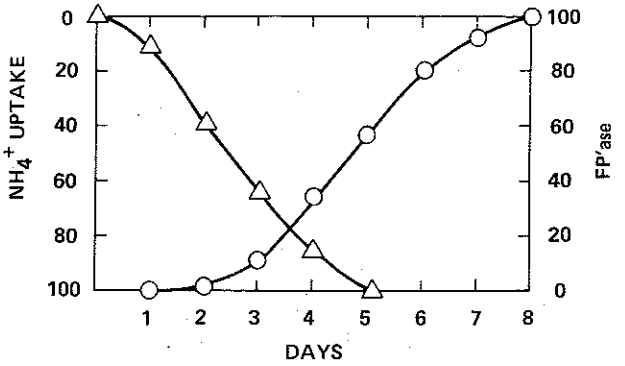

Fig. 4. Relative values for (△) ammonia uptake and (○) cellulase appearance in the filtrate. Cultured on 8% cellulose with revised medium.

Saccharification Potential

To evaluate the activity of the cellulase present in the filtrates from 8% cellulose fermentations and to demonstrate their capability in practical saccharifications, the filtrates were used to saccharify 15% slurries of BW 200 at four enzyme dilutions (Fig. 5). With the culture filtrate diluted to 0.12 (of its full strength), a 5% sugar syrup (based on total sugars) was produced in 24 hr, and 5% glucose was produced from 0.3 strength enzyme in 24 hr. Comparing these and

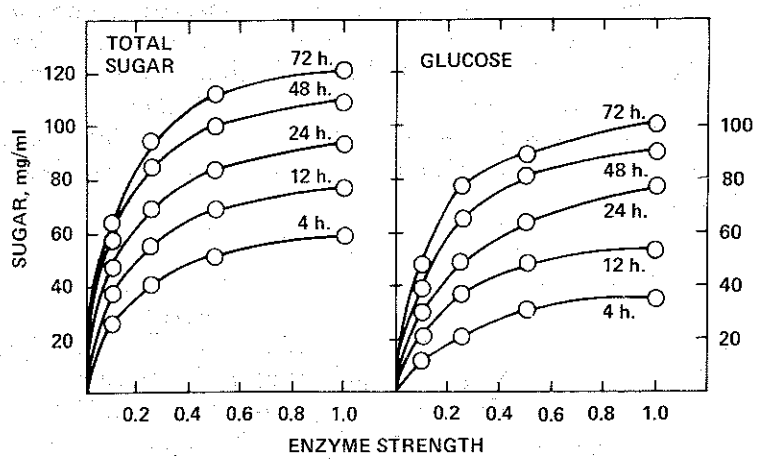

Fig. 5. Saccharification potential of a culture filtrate from 8% cellulose fermentation using the revised medium. Filtrate used at 0.1, 0.25, 0.50, and 1.0 times full strength to saccharify 15% BW 200. Samples taken at 4–72 hrs.

other values with those reported previously from fermentations on 0.75 and 2.0% cellulose,[5] the enzyme from the 8% cellulose fermentation was 3.3-fold that from 2% and 7-fold the strength from 0.75% cellulose fermentations based on total sugar. These values are in reasonable agreement with increases in both soluble protein and filter paper cellulase.

DISCUSSION

To culture a microorganism in stirred-tank fermentors with high cellulose concentrations, it is necessary to use a cellulose having a high bulk density. With the fermentors used in these studies the highest concentration of hammer-milled Solka Floc (SW 40) usable is only 2% because at higher levels the viscosity of the medium prohibits adequate mixing. Ball-milled Solka Floc, with its higher bulk density, permits easy handling in fermentors at least up to 8%. However, at these higher concentrations of BW 200 it appears necessary to start the fermentation with a 20% inoculum to avoid an early sluggish growth period and a poor cellulase yield.

At higher cellulose concentrations more nitrogen is required. In the past, nitrogen levels were raised by increasing the concentration of peptone and $(NH_4)_2SO_4$,[5-7] but this results in a higher level of unwanted soluble material in the filtrate at the end of the fermentation; if crude enzyme is used for saccharification, the salts will be present in the syrups. By using NH_4OH to control pH, the required nitrogen is added during the course of the fermentation. In the fermentation of 8% cellulose, 90% of the NH_4^+ consumed is provided by the added NH_4OH. Salt concentration in the medium can be further lowered by eliminating KH_2PO_4, which is used as a buffer for flask cultures in which pH cannot be automatically controlled. With a lower K^+ concentration in the medium, residual NH_4^+ is maintained at a higher level. Perhaps with less K^+ present more NH_4^+ can become associated with the phosphate ion. At any rate, cellulase production appears to be linearly related to cellulose concentrations up to 8%, and this linearity is attained in media which have a lower salt concentration than the standard Mandels-Reese medium.

The rise in pH after NH_4^+ consumption ceases appears to be due to the secretion of ammonia by the fungus. Mou and Cooney[14] have observed a similar relationship between ammonia nitrogen and pH in cellulose fermentation with *Trichoderma* in which the decomposition of urea was responsible for a rise in NH_4^+. In the fermen-

tations reported here the rise of NH_4^+ may result from the organism catabolizing its internal protein and secreting ammonia resulting from deamination, a phenomenon observed in starved cells of *Arthrobacter*.[15] This suggestion is supported by the observations of Mandels and Andreotti[6] on *T. reesei* cultured on cellulose in which mycelial protein content decreases in the latter part of the fermentation while cellulase activity and pH increase.

The carbon-to-nitrogen ratio (i.e., 15) for fermentation of 8% cellulose appears to be higher than the ratios (i.e., 12) for fermentations of lower cellulose concentrations. The reason for the higher C:N ratio may be that a significant portion of the cellulose is not consumed at the time NH_4^+ uptake ceases and protein catabolism starts. This appears more likely if the residual cellulose is of a highly crystalline nature causing it to be resistant to hydrolysis.

The relationship between the amount of cellulase in the medium and NH_4^+ uptake (Fig. 4) indicates that much of the cellulase is released after net assimilation of nitrogen ceases. This suggests that either cellulase has been synthesized earlier in the fermentation and is released later, or that a significant portion of the cellulase is synthesized from the turnover of preexisting cellular protein. The relationship between NH_4^+ uptake and cellulase is similar to that previously reported for cellulose consumption and cellulase production on 1% cellulose.[16] Berg and Pettersson[17] using *T. viride* claim that all the cellulase is synthesized by the time growth ceases and that most of the enzyme is cell bound and then released during starvation. However, in their system a relatively low concentration (0.5%) of very susceptible cellulose was used as the substrate and was consumed within a 24 hr period. These conditions are quite different from those presented here in which high initial concentrations of cellulose are consumed over a period of days. In any case the question of when cellulase is synthesized is important if one is to maximize enzyme productivity.

The authors thank Dr. Mary Mandels, Dr. Gabriel Mandels, and Dr. Elwyn Reese for their guidance during this work, which was funded by Interagency Agreement No. E(49-28)-1007 with the U.S. Department of Energy (formerly U.S. Energy Research and Development Administration).

References

1. M. Mandels and E. T. Reese, *J. Bacteriol.*, **73**, 269 (1957).
2. D. Sternberg, *Appl. Environ. Microbiol.*, **31**, 648 (1976).
3. D. E. Brown and D. J. Halsted, *Biotechnol. Bioeng.*, **17**, 1199 (1975).

4. M. Linko, P. Markkanen, M. Bailey, and M. Leisola, *Proceeding on Bioconversion Symposium*, T. K. Ghose, Ed. (IIT Delhi, New Delhi, India, 1977), p. 329.
5. D. Sternberg, *Biotechnol. Bioeng.*, **18,** 1751 (1976).
6. M. Mandels and R. E. Andreotti, *Proc. Biochem.*, **13,** 6 (1978).
7. J. M. Nyström and P. H. DiLuca, in Ref. 4, p. 293.
8. D. E. Brown, D. J. Halsted, and P. Howard, *Symposium on Enzymatic Conversion of Cellulose*, M. Bailey, T. M. Enari, and M. Linko, Eds. (SITRA, Helsinki, Finland, 1975), p. 137.
9. E. Simmons, abstracts, "Second International Mycological Congress," Tampa, Florida, 1977, p. 618.
10. J. M. Nyström and A. Allen, *Biotechnol. Bioeng. Symp.*, **6,** 55 (1976).
11. M. Mandels, R. Andreotti, and C. Roche, *Biotechnol. Bioeng. Symp.*, **6,** 21 (1976).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193,** 265 (1951).
13. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, **28,** 350 (1956).
14. D.-G. Mou and C. L. Cooney, *Biotechnol. Bioeng.*, **18,** 1371 (1976).
15. C. W. Boylen and J. C. Ensign, *J. Bacteriol.*, **103,** 578 (1970).
16. R. E. Andreotti, M. Mandels, and C. Roche, in Ref. 4, p. 249.
17. B. Berg and G. Pettersson, *J. Appl. Bacteriol.*, **42,** 65 (1977).

Accepted for Publication May 3, 1978